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Title of Invention:

Method of measuring protease inhibitors, a measurement kit using it, and a method of dissolving substrates

Abstract:

Purpose of Invention: To provide a method of manufacturing urinary trypsin inhibitors which has an excellent measurement accuracy and reproducibility, is easy to operate, and does not pose any risk of harming plastic cells.

Means of Solving the Problems of This Invention: A method of measuring urinary trypsin inhibitors by mixing the urine sample, an enzyme solution containing trypsin, and a buffer, adding a substrate solution to this mixture to perform an enzyme reaction, and measuring the enzyme activity. As the aforementioned buffer, a buffer prepared in such a way that it contains calcium in the ranges of 0.15 μmol or more per μg of the trypsin in the reaction solution and 100 μmol or less per ml of the aforementioned urine sample, and, when the aforementioned substrate solution is prepared by dissolving the substrate in an organic solvent and diluting this solution with water, at least one amphoteric or nonionic surface active agent is added to either or both the aforementioned organic solvent and water.

Claims:

(1) A method of measuring protease inhibitors which is a method in which a protease inhibitor in a sample is measured by compounding and mixing the sample, a protease, calcium, and a substrate and measuring the enzyme activity of the aforementioned enzyme; the proportion of the aforementioned calcium is in the ranges of 0.15 μmol or more per μg of the aforementioned enzyme and 100 μmol or less per ml of the aforementioned urine sample; the method of compounding the aforementioned substrate is that a substrate solution is prepared by dissolving the aforementioned substrate in an organic solvent, diluting this solution with water; and when this dilution is performed, at least one amphoteric or nonionic surface active agent is added to either or both the aforementioned organic solvent and the water.

(2) A measurement method in accordance with Claim (1), in which a buffer solution is used instead of water in preparing the substrate solution.

(3) A measurement method in accordance with Claim (1) or (2), in which the organic solvent used in preparing the substrate solution is dimethyl sulfoxide.

(4) A measurement method in accordance with any of Claims (1)–(3), in which the protease is trypsin and the substrate is shown by formula (1) below.

$$\text{protective group} - (\text{amino acid residual group})_n - \text{p-nitroanilide} \quad (1)$$

(where n is an integer in the range 1–5)

(5) A measurement method in accordance with Claim (4), in which the substrate is α-benzoyl-arginine-p-nitroanilide.

(6) A measurement method in accordance with Claim (4) or (5), in which the sample is a urine sample and the protease inhibitor is a urinary trypsin inhibitor.

(7) A measurement method in accordance with any of Claims (1)–(6), in which the surface active agent is a betaine-type amphoteric surface active agent.

(8) A measurement method in accordance with any of Claims (1)–(7), in which the amphoteric surface active agent is at least one of the following: 3-[(3-colamidepropyl)dimethylammonio]-1-propanesulfonic acid and 3-[(3-colamide-propyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid.

(9) A measurement method in accordance with any of Claims (1)–(7), in which the nonionic surface active agent is at least one of the following: polyoxyethyle-nesorbitan monolaurate, polyoxyethylenesorbitan monooleate, polyoxyethylene (23) lauryl ether, polyoxyethylene (20) cetyl ether, polyoxyethylene (10) octylphe-nyl ether, polyoxyethylene nonylphenyl ether, polyoxyethylene alkyl ethers, perfluoroalkyl polyoxyethylene ethanols, alkyl fluoride esters, polyethylene-glycol mono-p-nonylphenyl ether, polyoxyethylene (30) octylphenyl ether, N,N-bis(3-D-gluconamidepropyl) deoxycolamide, n-octyl-β-d-thioglucoside, and sucrose monolaurate.

(10) A measurement method in accordance with any of Claims (1)–(9), in which the proportions of the ingredients of the substrate solution, with respect to the total quantity of the solution, are 1–50 mmol/l substrate, 1–50 wt % organic solvent, and 0.1–5 wt % surface active agent.

(11) A kit for measuring protease inhibitors, which is a measurement kit for protease inhibitors provided with a protease, a substrate, and calcium, and in which the proportion of the aforementioned calcium is 0.15 mmol or more per mg of the aforementioned enzyme and 100 mmol or less per ml sample; the aforementioned substrate is dissolved in a solution; this solution contains an organic solvent and a surface active agent; and the aforementioned surface active agent is at least one amphoteric or nonionic surface active agent.

(12) A measurement kit in accordance with Claim (11), in which the solution containing the substrate is prepared by dissolving the substrate in an organic solvent and diluting this solution with water, and the aforementioned surface active agent is compounded with either or both the aforementioned organic solvent and water.

(13) A measurement kit in accordance with Claim (11) or (12), in which, when the reaction solution is prepared by compounding the protease, the substrate, the calcium, and the sample, the pH of this reaction solution is in the range of 5–9, the concentration of the aforementioned enzyme in the aforementioned reaction solution is in the range of 5–250 mg/l, and the substrate concentration in the aforementioned reaction solution is 0.5–25 mmol/l.

(14) A measurement kit in accordance with any of Claims (11)–(13), in which the organic solvent of the solution in which the substrate is dissolved is dimethyl sulfoxide.

(15) A measurement kit in accordance with any of Claims (11)–(14), in which the protease is trypsin and the substrate is shown by formula (2) below.

$$\text{protective group} - (\text{amino acid residual group})_n - \text{p-nitroanilide} \qquad (2)$$

(where n is an integer in the range 1–5)

(16) A measurement kit in accordance with Claim (15), in which the substrate is α-benzoyl-arginine-p-nitroanilide.

(17) A measurement kit in accordance with Claim (11)– (16), in which the surface active agent of the solution in which the substrate is dissolved is a betaine-type amphoteric surface active agent.

(18) A measurement kit in accordance with any of Claims (11)–(17), in which the amphoteric surface active agent of the solution in which the substrate is dissolved is at least one of the following: 3-[(3-colamidepropyl)dimethylammonio]-1-propanesulfonic acid and 3-[(3-colamidepropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid.

(19) A measurement kit in accordance with any of Claims (11)–(18), in which the nonionic surface active agent of the solution in which the substrate is dissolved is at least one of the following: polyoxyethylenesorbitan monolaurate, polyoxyethylenesorbitan monooleate, polyoxyethylene (23) lauryl ether, polyoxyethylene (20) cetyl ether, polyoxyethylene (10) octylphenyl ether, polyoxyethylene nonylphenyl ether, polyoxyethylene alkyl ethers, perfluoroalkyl polyoxyethylene ethanols, alkyl fluoride esters, polyethyleneglycol mono-p-nonylphenyl ether, polyoxyethylene (30) octylphenyl ether, N,N-bis(3-D-gluconamidepropyl) deoxycolamide, n-octyl-β-d-thioglucoside, and sucrose monolaurate.

(20) A measurement kit in accordance with any of Claims (11)–(19), which is provided with the buffer R1, the enzyme solution R2, and the substrate solution R3 mentioned below, in the volume proportions of R1:R2:R3 = 30–90:5–40:5–30.

(R1) A buffer solution containing calcium in the ranges of 0.15 μmol or more per μg of the aforementioned enzyme and 100 μmol or less per ml of the sample.

(R2) An enzyme solution containing the protease.

(R3) A substrate solution containing the substrate, an organic solvent, and a surface active agent; the aforementioned surface active agent is at least one amphoteric surface active agent or nonionic surface active agent.

(21) A method of dissolving the substrate, in which the substrate is dissolved in an organic solvent and this solution is diluted with water, and at least one amphoteric surface active agent or nonionic surface active agent is added to either or both the aforementioned organic solvent or the water.

(22) A method of dissolving the substrate in accordance with Claim (21), in which the dilution is performed with a buffer solution instead of water.

(23) A method of dissolving the substrate in accordance with Claim (21) or (22), in which the organic solvent used in preparing the substrate solution is dimethyl sulfoxide.

(24) method of dissolving the substrate in accordance with any of Claims (21)–(23), in which the substrate is shown by formula (3) below.

protective group – (amino acid residual group)$_n$ – p-nitroanilide (3)

(where n is an integer in the range 1–5)

(25) A method of dissolving the substrate in accordance with any of Claims (21)–(24), in which the surface active agent is a betaine-type amphoteric surface active agent.

(26) A measurement method in accordance with any of Claims (21)–(25), in which the amphoteric surface active agent is at least one of the following: 3-[(3-colamidepropyl)dimethylammonio]-1-propanesulfonic acid and 3-[(3-colamidepropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid.

(27) A measurement method in accordance with any of Claims (21)–(26), in which the nonionic surface active agent is at least one of the following: polyoxyethylenesorbitan monolaurate, polyoxyethylenesorbitan monooleate, polyoxyethylene (23) lauryl ether, polyoxyethylene (20) cetyl ether, polyoxyethylene (10) octylphenyl ether, polyoxyethylene nonylphenyl ether, polyoxyethylene alkyl ethers, perfluoroalkyl polyoxyethylene ethanols, alkyl fluoride esters, polyethyleneglycol mono-p-nonylphenyl ether, polyoxyethylene (30) octylphenyl ether, N,N-bis(3-D-gluconamidepropyl) deoxycolamide, n-octyl-β-d-thioglucoside, and sucrose monolaurate.

(28) A method of dissolving the substrate in accordance with any of Claims (21)–(27), in which the proportions of the ingredients of the substrate solution, with respect to the total quantity of the solution, are 1–50 mmol/l substrate, 1–50 wt % organic solvent, and 0.1–5 wt % surface active agent.

Detailed Explanation of Invention:

Industrial Field of Application

This invention concerns a method of measuring protease inhibitors, a measurement kit using this method, and a method of dissolving substrates.

Prior Art

Recently, urinary trypsin inhibitors, including urinary trypsin inhibitors (UTI), have been noted as indicators of the condition of the body, and various studies have been performed on them in the field of clinical medicine. For example, it is known that the aforementioned UTI appear in the urine when the body is exposed to endogenous or exogenous stresses, such as inflammations and surgery (T. Kuwajima et al., *Nyōchū toripushin inhibitā no rinshōteki igi* [The clinical significance of urinary trypsin inhibitors], *Japanese Journal of Inflammation*, review article, Vol. 9, No. 3, May 1989).

Since the aforementioned urinary tripsin inhibitors inhibit trypsin activity in correspondence with their quantity, they are measured by measuring the degree of inhibition of trypsin activity. This measurement can be performed, for example, by a method of mixing a urine sample, an enzyme solution containing trypsin, and a buffer, adding a substrate solution to this, and measuring the enzyme reaction.

In this measurement, benzoyl-arginine-p-nitroanilide (BAPNA) can be used as the substrate. However, since BAPNA is difficult to dissolve, the substrate solution is prepared by first dissolving BAPNA in dimethylsulfoxide (DMSO) and diluting this solution approximately 2 times with water. Moreover, when this measurement is performed, calcium, a trypsin activator, is used; ordinarily, the calcium is compounded with the aforementioned buffer.

Problems That the Invention Is to Solve

However, the conventional measuring method has the following problems.

First, if the calcium concentration in the buffer, etc., is low, the effect of the calcium present in the urine sample will be felt from the beginning, and as a result, the trypsin will be activated and the value measured will be lower than the true urinary trypsin inhibitor concentration. Moreover, if an excess quantity of calcium is added, it will react with the carbonate, phosphate, etc., ions in the urine and produce a precipitate, which will affect the measurement. In order to prevent this, a pre-treatment, such as centrifuging, may be performed, but the measurement operation will become more complex.

Next, there is a risk that the organic solvent, such as DMSO, may harm the plastic cells which are generally used in automatic analyzers; therefore, the quantity of the organic solvent used is limited. Consequently, the quantity of the substrate which can be dissolved is also limited, and as a result it becomes more difficult to increase the measurement sensitivity, and there are limits on the simultaneous reproducibility of the measurement. Furthermore, there is a risk that the activity of the trypsin will be limited by the use of the organic solvent. In addition, it is possible to dissolve BAPNA, which is difficult to dissolve, by using an organic solvent, but the results of this use are not sufficient, and when the substrate solution is stored in a cold place for a long period, there is a risk that the BAPNA will crystallize out. Therefore, in the conventional measurement method, when difficult-to-dissolve substances such as BAPNA are employed by

using organic solvents, the substrate solution must be prepared each time it is needed for a measurement, and the measurement must be performed immediately.

Therefore, the purpose of this invention is to provide a method of manufacturing urinary trypsin inhibitors which has an excellent measurement accuracy and simultaneous reproducibility, is easy to operate, and does not pose any risk of harming plastic cells, as well as a measurement kit which uses this method, and a method of dissolving substrates.

Means of Solving These Problems

In order to accomplish this purpose, the method of measuring protease inhibitors of this invention is a method of measuring protease inhibition in samples by mixing the sample, a protease, calcium, and a substrate and measuring the enzyme activity of the aforementioned enzyme. The ranges of the aforementioned calcium are 0.15 μmol or more per μg of the trypsin in the reaction solution and 100 μmol or less per ml of the aforementioned sample. The method of compounding the aforementioned substrate [solution] is to dissolve the substrate in an organic solvent and dilute this solution with water to form the substrate solution. When this dilution is performed, at least one amphoteric or nonionic surface active agent is added to either or both the aforementioned organic solvent and water.

In the measurement method of this invention, in this manner, the proportion of the calcium used is defined, and specific surface active agents are used together with the organic solvent in compounding the substrate [solution]. That is, if the calcium content in the reaction solution is 0.15 μmol or more per μg of the trypsin, the trypsin activity is made constant, so that there is no danger that the effect of the calcium in the urine will be felt. Furthermore, if the calcium content in the reaction solution is 100 μmol or less per ml of the urine sample, a precipitate will not be produced and there is no danger of bad effects on the measurement; in addition, difficult operations such as centrifugation can be eliminated. Furthermore, by using the aforementioned specific surface active agents, the quantity of the organic solvent, such as DMSO, used can be reduced and sufficient quantities of substrates which are difficult to dissolve, such as BAPNA, can be used. As a result, since the quantity of the organic solvent is small, damage to the plastic cells can be prevented, and since a sufficient quantity of substrate can be used, the measurement accuracy is increased and simultaneous reproducibility is improved. Furthermore, the solubility of the substrate can be improved, and crystallization can be prevented, by using specific surface active agents.

In the method of measurement of this invention, one can also use a buffer in place of water in the preparation of the substrate solution, and it is desirable to use DMSO as the organic solvent.

In the measurement method of this invention, it is desirable for the protease to be trypsin, and for the substrate to be the substrate shown by the aforementioned formula. As the aforementioned substrate, α-benzoyl-arginine-p-nitro-

anilide is especially desirable. However, one can also use α-benzoyl-lysine-p-nitroanilide, t-butoxycarbonyl-arginine-p-nitroanilide, and t-butoxycarbonyl-lysine-p-nitroanilide. In this case, moreover, it is desirable for the sample to be a urine sample and for the protease inhibitors to be urinary trypsin inhibitors.

In the measurement method of this invention, it is desirable for the surface active agents to be betaine-type surface active agents.

In the measurement method of this invention, it is desirable for the amphoteric surface active agent to be at least one of the following: 3-[(3-colamidepropyl)dimethylammonio]-1-propanesulfonic acid and 3-[(3-colamidepropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonic acid.

In the measurement method of this invention, it is desirable for the nonionic surface active agent to be at least one of the following: polyoxyethylenesorbitan monolaurate, polyoxyethylenesorbitan monooleate, polyoxyethylene (23) lauryl ether, polyoxyethylene (20) cetyl ether, polyoxyethylene (10) octylphenyl ether, polyoxyethylene nonylphenyl ether, polyoxyethylene alkyl ethers, perfluoroalkyl polyoxyethylene ethanols, alkyl fluoride esters, polyethyleneglycol mono-p-nonylphenyl ether, polyoxyethylene (30) octylphenyl ether, N,N-bis(3-D-gluconamidepropyl) deoxycolamide, n-octyl-β-d-thioglucoside, and sucrose monolaurate. Examples of the aforementioned polyoxyethylene nonylphenyl ether are Noigen EA-80, Noigen EA-120, and Noigen EA-140 (all products of Daiichi Kogyo Pharmaceutical Co.). Examples of the aforementioned polyoxyethylene alkyl ethers are Softanol [*sofutanōru*] 70, Softanol 90, and Softanol 120 (all products of Nihon Shokubai Co.). An example of the aforementioned perfluoroalkyl polyoxyethylene ethanols is Florad [*furoraado*] FC-170C (3M Co.), and an example of the aforementioned alkyl fluoride esters is Florad FC-430 (3M Co.). An example of the aforementioned polyoxyethylene (30) octylphenyl ether is Triton X-305 (Nakarai Tesuku Co.).

In the measurement method of this invention, the proportions of the ingredients of the substrate solution should be are 1–50 mmol/l substrate, 1–50 wt % organic solvent, and 0.1–5 wt % surface active agent with respect to the total quantity of the substrate.

Next, the kit for measuring protease inhibitors of this invention is a protease inhibitor measurement kit provided with a protease, a substrate, and calcium; the proportion of the aforementioned calcium is 0.15 μmol or more per μg of the aforementioned enzyme and 100 μmol or less per ml of the sample; the aforementioned substrate is dissolved in the solution, and this solution contains an organic solvent and a surface active agent. The aforementioned surface active agent is at least one amphoteric or nonionic surface active agent.

By using this measurement kit, protease inhibitors can be measured in a simple manner, with excellent measurement accuracy and simultaneous reproducibility, and with no risk of damaging the plastic cells.

In the measurement kit of this invention, the solution in which the aforementioned substrate is dissolved is prepared by diluting the substrate in an organic

solvent and diluting this solution with water; it is desirable for either or both the aforementioned organic solvent and water to contain a surface active agent.

In the measurement kit of this invention, the reaction solution is prepared by compounding together the protease, the substrate, the calcium, and the sample; the pH of this reaction solution is in the range of 5–9, the concentration of the aforementioned enzyme in the aforementioned reaction solution is in the range of 5–250 mg/l, and the substrate concentration in the aforementioned reaction solution is 0.5–25 mmol/l.

In the measurement kit of this invention, it is desirable to use DMSO as the organic solvent and to use a substrate shown by formula (2) above as the substrate. It is especially desirable to use α-benzoyl-arginine-p-nitroanilide as the substrate.

In the measurement kit of this invention, it is desirable to use the same surface active agents as were mentioned above concerning the measurement method of this invention.

A desirable form of the measurement kit of this invention is one which contains the buffer R1, the enzyme solution R2, and the substrate solution R3 mentioned below, in the volume proportions of R1:R2:R3 = 30–90:5–40:5–30.

(R1) A buffer solution containing calcium in the ranges of 0.15 μmol or more per μg of the aforementioned enzyme and 100 μmol or less per ml of the sample.

(R2) An enzyme solution containing the protease.

(R3) A substrate solution containing the substrate, an organic solvent, and a surface active agent; the aforementioned surface active agent is at least one amphoteric surface active agent or nonionic surface active agent.

Furthermore, in the measurement kit of this invention, the calcium may be contained in the aforementioned enzyme solution R2 or the substrate solution R3 instead of the aforementioned buffer R1, as long as it has the specific concentration measured above. Furthermore, the calcium may also be contained, in the aforementioned specific concentration, partly in the aforementioned buffer R1 and partly in the aforementioned enzyme solution R2, or partly in the buffer R1 and partly in the substrate solution R3, or partly in the enzyme solution R2 and partly in the substrate solution R3, or partly in the buffer R1, partly in the enzyme solution R2, and partly in the substrate solution R3. Moreover, in the measurement kit of this invention, the aforementioned R1, R2, and R3 may be independent of each other, or it may combine a mixture of any two of the solutions with the third solution. Specifically, the following three combinations may be used:

(1) A mixture of R1 and R2 + R3

(2) A mixture of R1 and R3 + R2

(3) A mixture of R2 and R3 + R1

In combination (3), furthermore, the enzyme and the substrate can be mixed, if the enzyme concentration is controlled by regulating the pH, etc.

Next, the method of dissolving the substrate of this invention is a method in which the substrate is dissolved in an organic solvent and this solution is diluted with water; at least one amphoteric surface active agent or nonionic surface active agent is added to either or both the aforementioned organic solvent or the water.

The method of dissolving the substrate of this invention is not limited to the aforementioned protease substrates; it can be applied to the dissolution of various kinds of substrates.

In the method of dissolving the substrate of this invention, a buffer may be used instead of the water, in the same manner as was mentioned above, and it is desirable to use DMSO as the organic solvent.

In the method of dissolving the substrate of this invention, the substrates and surface active agents which are desirable for use are the same as those mentioned above.

In the method of dissolving the substrate of this invention, the proportions of the ingredients of the substrate solution, with respect to the total quantity of the solution, are 1–50 mmol/l substrate, 1–50 wt % organic solvent, and 0.1–5 wt % surface active agent.

Practical Form of This Invention

Next, this invention will be explained in more detail.

The method of measuring protease inhibitors of this invention can be performed, for example by using a protease solution, a substrate solution prepared by using an organic solvent and specific surface active agents, and a buffer solution with a specific calcium concentration range.

The enzyme mentioned above may be, for example, trypsin. This trypsin is not particularly limited; for example, one can use bovine pancreatic trypsin, porcine pancreatic trypsin, etc. A suitable trypsin concentration is determined by the specific activity of the trypsin, but it is ordinarily 10–500 mg/l, preferably 20–100 mg/l, with respect to the total enzyme solution. Moreover, the pH of this enzyme solution may be adjusted to 2.0–3.0 with hydrochloric acid or a buffer in order to prevent autodigestion of the trypsin.

Furthermore, chymotrypsin may also be used as another protease. An example of a substrate which can be used with chymotrypsin is benzoyl-tyrosine-p-nitroanilide.

Next, the surface active agents used in the aforementioned substrate solution are, as mentioned above, amphoteric and/or nonionic surface active agents. Preferable surface active agents are as mentioned above; especially desirable ones, from the point of view of obtaining still more excellent effects from this invention, are 3-[(3-colamidepropyl)dimethylammonio]-1-propanesulfonic acid

(CHAPS) and 3-[(3-colamidepropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPSO). In this invention, the aforementioned surface active agents may be used individually or in combinations of 2 or more kinds.

Moreover, as the substrate of this substrate solution, as mentioned above, substrates shown by the aforementioned formula (1) are desirable, and the aforementioned α-benzoyl-arginine-p-nitroanilide is especially desirable.

Furthermore, as the aforementioned organic solvent, besides the aforementioned DMSO, one can use, for example, dimethylformamide (DMF).

In this invention, the organic solvent in which the substrate is dissolved is diluted with water or a buffer. In this dilution, whether one uses water or a buffer, and which kind of buffer is used, is decided by the measurement conditions, etc. Examples of buffers which can be used are triethanolamine hydrochloride buffer solution, tris-hydrochloric acid buffer solution, phosphate buffer solution, glycine buffer solution, Veronal hydrochloric acid, Good's [*guddo*] buffer solution, etc. The pH of these buffer solutions is decided according to the kind of enzyme used, etc.

The preparation of the substrate solution of this invention is performed, for example, in the following manner. First, the substrate is dissolved in an organic solvent. The concentration at this time is ordinarily in the range of 1–50 mg per 1 mg DMSO. Otherwise, the aforementioned specific surface active agents may be dissolved in water or a buffer solution to prepare a surface active agent solution. The concentration in this case is determined by the kind of surface active agent used, but ordinarily it is in the range of 0.1–5 wt % of the water or buffer solution. Then, the substrate solution is prepared by diluting the aforementioned organic solvent with the aforementioned surface active agent solution. The dilution rate is ordinarily 2–20 times, preferably 10–20 times. Furthermore, the surface active agent is ordinarily compounded with the water or buffer solution, but it may also be compounded with the organic solvent.

Next, in this example, the calcium is added to the buffer solution; the concentration is in the range mentioned above, preferably 0.2 μmol or more per μg of the aforementioned enzyme, and 50 μmol or less per ml of the sample. The pH of this buffer solution should be within a range such that the aforementioned pH of the enzyme reaction solution is produced, preferably pH 7–8. Moreover, the kind of buffer solution used may be a triethanolamine hydrochloride buffer solution, tris-hydrochloric acid buffer solution, phosphate buffer solution, Good's buffer solution, etc. This buffer solution is prepared by an ordinary method.

Next, the method of measurement of this invention is performed in the following way, in the case in which urinary trypsin inhibitors, for example, are the object of measurement.

First, the urine sample, the buffer solution, and the enzyme solution are mixed. The proportions (weight ratios) in this case are ordinarily set within the ranges of urine solution : buffer solution : enzyme solution = 1:5–10:2–5. Next, this mixture is incubated; the incubation conditions are ordinarily 1–5 minutes at

25–37°C. The aforementioned substrate solution is then compounded with the result of this incubation, causing a reaction between the aforementioned enzyme and substrate. The proportion of this composition is ordinarily in the range of 5–30 vol % with respect to the whole reaction solution. The reaction conditions are ordinarily 1–10 minutes at 25–37°C. Moreover, the pH of the reaction solution at this time varies with the kind of enzyme used, etc., but in the case of trypsin, which is presently under discussion, it is in the range of pH 7–8. Furthermore, the enzyme reaction is detected by using a specific method, and the enzyme activity is measured. In this reaction, the enzyme reaction is inhibited corresponding to the quantity of trypsin inhibitors in the aforementioned urine sample. Therefore, by making a calibration curve beforehand, using known urinary trypsin inhibitors, the quantity of urinary trypsin inhibitors can be measured. The method of detecting the aforementioned enzyme reaction may be to measure the degree of color which is produced when the enzyme reaction occurs by means of a spectrophotometer, etc. Otherwise, the enzyme activity can be measured by measuring the concentration of the reaction product.

Next, the measurement kit of this invention is provided, for example, with the aforementioned buffer solution R1, enzyme solution R2, and substrate solution R3. The preparation of these reagents R1, R2, and R3, can be performed by the methods described in the explanation of the measurement method of this invention, and the compositions and their proportions are as described above. By using this measurement kit, the measurement of the protease inhibitors, such as urinary trypsin inhibitors, can be performed in a simple and rapid manner.

Next, in the method of dissolution of the substrate of this method, the substrates used may be, besides the examples given above, Z-glycine-glycine-leucine-p-nitroanilide or succinyl-alanine-alanine-alanine-p-nitroanilide. Moreover, the enzymes used in the method of dissolving the substrate in this invention are not particularly limited; one may use, for example, trypsin, chymotrypsin, elastase, subtilisin, plasmin, thrombin, kallikrein, cathepsin B, endopeptidase, or urikinase.

The method of dissolving the substrate of this invention and measuring it as the same as was described in the explanation of the measurement method given above.

Working Examples

Next, working examples of this invention will be explained.

Working Example 1

The buffer solution R1, enzyme solution R2, and substrate solution R3 were prepared as described below.

Buffer solution R1: The following ingredients were mixed with purified water in the proportions shown below to prepare a buffer solution (pH 7.8), by the ordinary method.

Triethanolamine hydrochloride	0.2 mol/l
$CaCl_2$	0.003 mol/l

Enzyme solution R2: The following ingredients were mixed in the proportions shown below to prepare an enzyme solution, by the ordinary method.

Bovine pancreatic trypsin (Type III, 10,000–13,000 BAEE units/mg, Sigma Co.)	50 mg/l
Hydrochloric acid	1.2 mmol/l

Substrate solution R3: The necessary quantity of BAPNA was dissolved in DMSO and the result was diluted 10 times with an aqueous solution of a specific surface active agent to prepare 4 kinds of substrate solution (a–d). Furthermore, for comparison, a substrate solution was prepared in the same manner except that no surface active agent was included. These compositions are shown below.

Substrate solution (a)

BAPNA	500 mg
DMSO	10 ml
CHAPSO	2.6 g
Purified water	90 ml

Substrate solution (b)

BAPNA	500 mg
DMSO	10 ml
CHAPSO	1.3 g
Purified water	90 ml

Substrate solution (c)

BAPNA	500 mg
DMSO	10 ml
CHAPS	2.6 g
Purified water	90 ml

Substrate solution (d)

BAPNA	500 mg
DMSO	10 ml
CHAPS	1.3 g
Purified water	90 ml

Substrate solution (comparison)

BAPNA	500 mg

DMSO	50 ml
Purified water	50 ml

Besides this, aqueous solutions of urinary trypsin inhibitor (UTI, Miraclid [*mirakuriddo*], Mochida Pharmaceutical Co.) were prepared with 3 concentrations, 0 U/ml, 100 U/ml, and 200 U/ml, and were used as samples.

Next, 0.14 ml of each sample, 1.8 ml buffer solution R1 and 0.48 ml enzyme solution R2 were mixed and kept at 37°C for 1 minute, after which 0.58 ml of the aforementioned substrate solution R3 was added and the reaction was started. The changes in absorbance (405 nm) within 100 seconds were measured by means of a spectrophotometer and relative absorbances (ΔO.D.) were obtained to make the calibration curves shown by the graph in Fig. 1.

From these results it can be seen that, if the specified surface active agents are used in preparing the substrate solution, the enzyme activity of the trypsin is increased. Furthermore, the enzyme activity is increased further by using a larger quantity of surface active agent in the composition.

Furthermore, in preparing the substrate solutions of this working example, the quantity of DMSO used can be reduced by using the specified surface active agents, a sufficient quantity of substrate can be dissolved, and crystallization and precipitation of the substrate can be prevented.

Working Example 2

Using urine samples from 5 healthy subjects (A, B, C, D, and E), and the same buffer solution R1, enzyme solution R2, and substrate solution R3 (a) as in Working Example 1, UTI measurements were performed 3 times in the same manner as in Working Example 1 and the UTI quantities were obtained by using the calibration curve from Working Example 1. The results are shown in Table 1.

	A	B	C	D	E
Measurement values (U/ml)	11.9	22.1	19.2	8.3	2.5
	11.1	22.3	19.2	7.7	4.7
	12.9	21.6	18.7	3.1	3.7

It can be seen from Table 1 that reliable UTI values could be obtained by this measurement. Furthermore, this measurement was not obstructed by precipitation, etc.

Working Example 3

Eighteen substrate solutions were prepared in the same manner as in the working examples given above by using, as the surface active agents, polyoxyethylenesorbitan monolaurate, polyoxyethylenesorbitan monooleate, polyoxy-

ethylene (23) lauryl ether, polyoxyethylene (20) cetyl ether, polyoxyethylene (10) octylphenyl ether, Softanol [*sofutanōru*] 70 (Nihon Shokubai Co.), Softanol 90 (Nihon Shokubai Co.), Softanol 120 (Nihon Shokubai Co.), Noigen EA-80 (Daiichi Kogyo Pharmaceutical Co.), Noigen EA-120 (Daiichi Kogyo Pharmaceutical Co.), Noigen EA-140 (Daiichi Kogyo Pharmaceutical Co.), Florad [*furoraado*] FC-170C (3M Co.), Florad FC-430 (3M Co.), polyethyleneglycol mono-p-nonylphenyl ether, Triton X-305 (Nakarai Tesuku Co.), N,N-bis(3-D-gluconamidepropyl) deoxycolamide, n-octyl-β-d-thioglucoside, and sucrose monolaurate. The composition of the substrate solution was as follows:

BAPNA	500 mg
DMSO	10 ml
Surface active agent	2.6 g
Purified water	90 ml

Next, the solubility of the substrate was investigated for the substrate solutions mentioned above. That is, when the aforementioned substrate solutions were left standing for 24 hours at 4°C, no crystallization and precipitation of the substrate was produced. From this result, it was concluded that sufficient quantities of the substrates can be dissolved with a low concentration of DMSO whichever of the aforementioned nonionic surface active agents was used. Furthermore, when a comparison experiment was performed by not adding a nonionic surface active agent, crystallization and precipitation of BAPNA were produced.

Next, among the substrate solutions prepared in this manner, the one in which polyoxyethylenesorbitan monolaurate was used was taken as the substrate solution R3. After a calibration curve was made in the same manner as in Working Example 1, the UTI quantity was measured for a human urine sample in the same manner as in Working Example 2; as a result, the UTI quantity was 29.0 U/ml. In this measurement, no precipitate was produced, and the UTI quantity obtained was reliable.

Effects of Invention

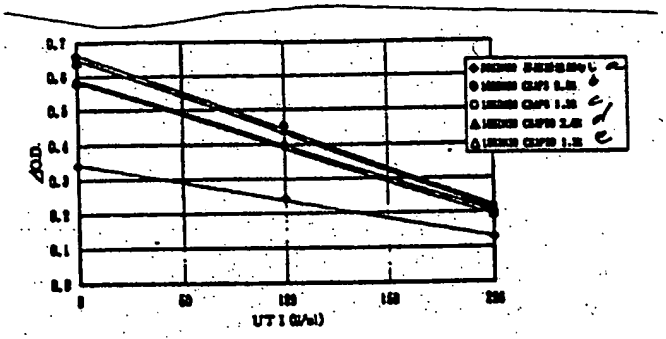
As discussed above, by using the method of measuring protease inhibitors of this invention, it is possible to obtain excellent measurement accuracy and simultaneous reproducibility; the operation of the method is simple, and there is no risk of harming the plastic cells. That is, since the calcium concentration is specified, a lower value than the true one is not obtained, and no precipitate, which causes measurement error, is produced. Furthermore, by using the specified surface active agents, the quantity of organic solvent used, which has a bad effect on the enzyme activity of the trypsin, etc., can be reduced, and a sufficient quantity of substrate can be added. Furthermore, since the enzyme activity of the trypsin, etc., is higher than in the prior art, the measurement sensitivity is improved, as well as the simultaneous reproducibility. In addition, since no precipitate is produced, pre-treatments such as centrifugation are not needed, and since the solubility of the substrate is improved, a large quantity of substrate solution can be prepared at one time, and the operation of the method is simplified when

compared with the prior art, in which it needed to be prepared specially for each measurement. Furthermore, by using the measurement kit of this invention, the work involved in preparing each reagent can be reduced and measurements can be performed in a short time and in a simple manner. In addition, the method of dissolving the substrate in this invention is not limited to substrates of proteases such as trypsin; it can also be applied to various other kinds of substrates.

Brief Explanation of Drawings

Fig. 1: graph showing calibration curves of UTI in the first working example of this invention.

Fig. 1



a. 50% DMSO; no surface active agent
b. 10% DMSO: 2.6% CHAPS
c. 10% DMSO; 1.3% CHAPS
d. 10% DMSO; 2.6% CHAPSO
e. 10% DMSO; 1.3% CHAPSO

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